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Biosynthesis and secretion of mouse cysteine-rich with EGF-like domains 2

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ABSTRACT

In this study, we found that Cysteine-rich with EGF-like domains 2 (CRELD2), a novel endoplasmic reticulum stress-inducible protein, is not only localized in the ER-Golgi apparatus but also spontaneously secreted. Deletion of four C-terminal amino acids from mouse CRELD2 or addition of tag-peptides to its C-terminus dramatically enhanced CRELD2 secretion. Intra- and extra-cellular CRELD2 is differentially glycosylated and its spontaneous secretion was significantly prevented by overexpression of a dominant negative mutant Sar1 and treatment with brefeldin A. Overexpression of wild-type GRP78 remarkably enhanced the secretion of wild-type but not mutant CRELD2. Our results demonstrate both that CRELD2 is a novel secretory glycoprotein regulated by Sar1 and GRP78 and that the C-terminal of CRELD2 plays a crucial role in its secretion.

1. Introduction

The endoplasmic reticulum (ER) is an organelle that plays a key role in folding and modifying newly synthesized transmembrane and secretory proteins [1,2]. Some pathophysiological conditions are reported to disrupt ER function by accumulating unfolded proteins [3,4]. Abnormal function due to their accumulation is referred to as ER stress, and various stress response phenomena are mediated by three resident ER stress sensors: PERK [5], IRE [6] and ATF6 [7]. Many genes have been identified as ER stress-inducible ones, and the stress sensors were demonstrated to regulate their expression [5–10].

Using microarray analysis of thapsigargin-inducible genes in Neuro2a cells, we recently identified *CRELD2* gene as a novel ER stress-inducible gene [11]. In addition, ATF6 has been found to positively regulate the transcription of the *CRELD2* gene through the well-conserved ER stress response element (ERSE) at the proximal region of the mouse CRELD2 promoter.

CRELD2 was first identified as a homologue to *CRELD1*, which is one of the atrioventricular septal defects (AVSD)-related genes [12–14]. CRELD2 has been reported to mediate the intracellular trafficking of acetylcholine receptor α4 and β2 subunits [15,16]. We previously reported that CRELD2 is an approximately 60-kDa glycoprotein that predominantly localizes to the ER and Golgi apparatus [11]. However, the molecular features of CRELD2 protein are poorly understood.

In this study, we first characterize that CRELD2 is a novel secretory glycoprotein and show that modification of its C-terminal region enhances its secretion. In addition, two factors, GRP78 [17] and Sar1 [18], are found to regulate the secretion of wild-type CRELD2. These results suggest that CRELD2 might act as a multifunctional factor both in the ER-Golgi apparatus and in the extracellular space under normal and pathophysiological conditions.

2. Materials and methods

2.1. Materials

Tunicamycin (Tm) and brefeldin A (BFA) were purchased from Sigma. Antibodies against Myc (9E10) and GRP78 were obtained from Santa Cruz Biotechnology. An antibody against CRELD2 was purchased from R&D Systems. Monoclonal Antibodies against human calnexin or human GM130 were obtained from MBL. Endoglycosidase H (Endo H) and *N*-Glycosidase F (*N*-Gly) were purchased from New England Biolabs and Roche Biochemicals, respectively.

2.2. Construction of plasmids

For preparation of mouse CRELD2 and GRP78 expression constructs, full-length mouse *CRELD2* and *GRP78* cDNAs were obtained from DNAFORM. Each type of Myc/His-tagged mouse *CRELD2* gene was amplified by PCR and then cloned into the pcDNA3.1/myc-His vector (FL-, Δ1 (1–287 aa)- and Δ2 (1–195 aa)-MH). Hemagglutinin (FL-HA)-tagged and Flag-tagged CRELD2 (ΔN (28–350 aa)-MH) were also prepared by PCR and cloned into the pcDNA3.1 vector and pFlag-CMV vector, respectively. Mouse wild-type

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GRP78 (wtGRP78) and GRP78 lacking four C-terminal amino acids (KDEL) (ΔGRP78) genes were also amplified by PCR using mouse *GRP78* cDNA and cloned into the pcDNA3.1 vector. HA-tagged Sar1 construct (H79G) were kindly provided by Dr. Wei Liu and Dr. Jennifer Lippincott-Schwartz [18].

2.3. Cell culture and treatment

COS7 cells and HEK293 cells were maintained in Dulbecco's Modified Eagle minimum essential Medium (DMEM) containing 8% fetal bovine serum. Transfection of each construct used in this study was performed using Lipofectamine-Plus reagent (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after transfection, culture medium was changed with fresh DMEM and the cells were incubated for the indicated time. To detect expression of Myc/His-tagged CRELD2 or other types of CRELD2 in culture medium, the cells were incubated in the presence or absence of serum, respectively. To evaluate the effects of Tm (2 μg/ml) or BFA (5 μg/ml) on CRELD2 processing and secretion, each reagent was added into the culture medium when culture medium was changed with fresh DMEM.

2.4. Western blotting analysis

Cells were lysed with homogenate butter [20 mM Tris–HCl (pH 8.0) containing 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A]. After protein concentration was determined, each cell lysate was dissolved in sodium dodecyl sulfate (SDS)–Laemmli sample buffer [62.5 mM Tris–HCl (pH 6.8), 2% SDS and 10% glycerol]. For detection of every type of CRELD2 in culture medium, an aliquot of CRELD2-containing culture medium was concentrated using Microcon (Millipore) or dried by a vacuum evaporator, and resuspended with SDS–Laemmli sample buffer. To detect Myc/His-tagged CRELD2 (CRELD2-MH) in culture medium, CRELD2-MH was concentrated by incubation with Ni-conjugated Sepharose (GE Healthcare Bioscience) at 4 °C for 2 h. After brief centrifugation, Ni-conjugated Sepharose was washed and resuspended with SDS–Laemmli sample buffer. In each experiment, equal amounts of each sample from lysates and culture medium were separated on 8.0% or 10.0% SDS–polyacrylamide electrophoresis gels, transferred onto polyvinylidene difluoride (PVDF) membrane (GE Healthcare Bioscience) and identified using primary antibodies against CRELD2, Myc-epitope or GRP78.

2.5. Fluorescent immunocytochemistry

COS7 cells expressing each type of CRELD2 on glass coverslips were fixed with PBS containing 4% paraformaldehyde for 10 min as described previously [19]. For the detection of Myc-tagged CRELD2 or untagged-CRELD2, fixed cells were permeabilized with PBS containing 0.2% Triton X-100 for 3 min. The coverslips were then incubated with an anti-Myc monoclonal antibody or an anti-CRELD2 polyclonal antibody for 1 h at room temperature. After washing, coverslips were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG or anti-goat IgG as a secondary antibody, for anti-Myc and anti-CRELD2, respectively. Cell nuclei were stained with Hoechst 33823. To characterize the subcellular localization of CRELD2 more precisely, cells expressing the indicated types of CRELD2 were stained with an anti-CRELD2 polyclonal antibody together with anti-calnexin or GM130 monoclonal antibody. For secondary antibodies, fluorescein isothiocyanate (FITC)-conjugated anti-goat IgG and rhodamine-conjugated anti-mouse IgG were used. In all cases, the cells were mounted in Vectashield (Vector Labs) and observed by fluorescence microscopy (KEYENCE).

2.6. Endoglycosidase digestions

An aliquot of cell lysate or culture medium in SDS–Laemmli sample buffer obtained from CRELD2-transfected cells was incubated with either Endo H (250 U) or *N*-Gly (0.5 unit) for 12 h at 37 °C, as described previously [20]. As a control, an equal amount of the same cell lysate was incubated without enzyme. Each sample was then analyzed by Western blotting using antibodies against CRELD2 or Myc-epitope, as described above.

3. Results and discussion

Previously, we identified the mouse *CRELD2* gene as a novel ER stress-inducible gene and observed that CRELD2-MH and EGFP-tagged CRELD2 are predominantly localized in the perinuclear region including the ER and Golgi apparatus [11]. CRELD2 was originally identified as a homolog of CRELD1 [12–14]. However, CRELD2 does not contain any putative transmembrane regions homologous to amino acid sequences of CRELD1. To characterize the molecular features of CRELD2, we first compared C-terminal amino acid sequences of CRELD2 among various species. As shown in Fig. 1A and B, four amino acids, (R/H)EDL, at the C-terminus of CRELD2, were well conserved among the seven indicated species

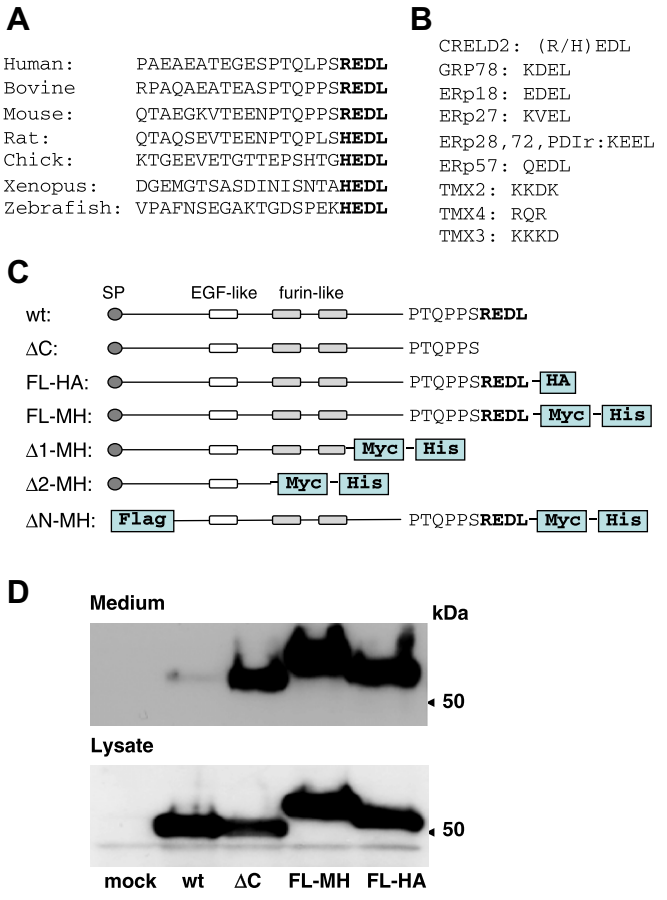

Fig. 1. Deletion of the C-terminal region of CRELD2 causes aberrant secretion. (A) Comparison of amino acid sequences of CRELD2 at the C-terminus among various species. (B) Four conserved C-terminal amino acids in various ER-resident proteins. (C) Schematic structure of mouse CRELD2 expression vectors used in this study. (D) Twenty-four hours after transfection of the indicated type of CRELD2 or an empty vector (mock) into HEK293 cells, the culture medium was replaced with serum free DMEM and the cells were incubated for an additional 18 h. The amount of CRELD2 in each lysate or in the culture medium was determined by western blotting, as described in Section 2. SP indicates a signal sequence at the N-terminus. Open and hatched boxes represent an EGF-like domain and furin-like repeats, respectively.

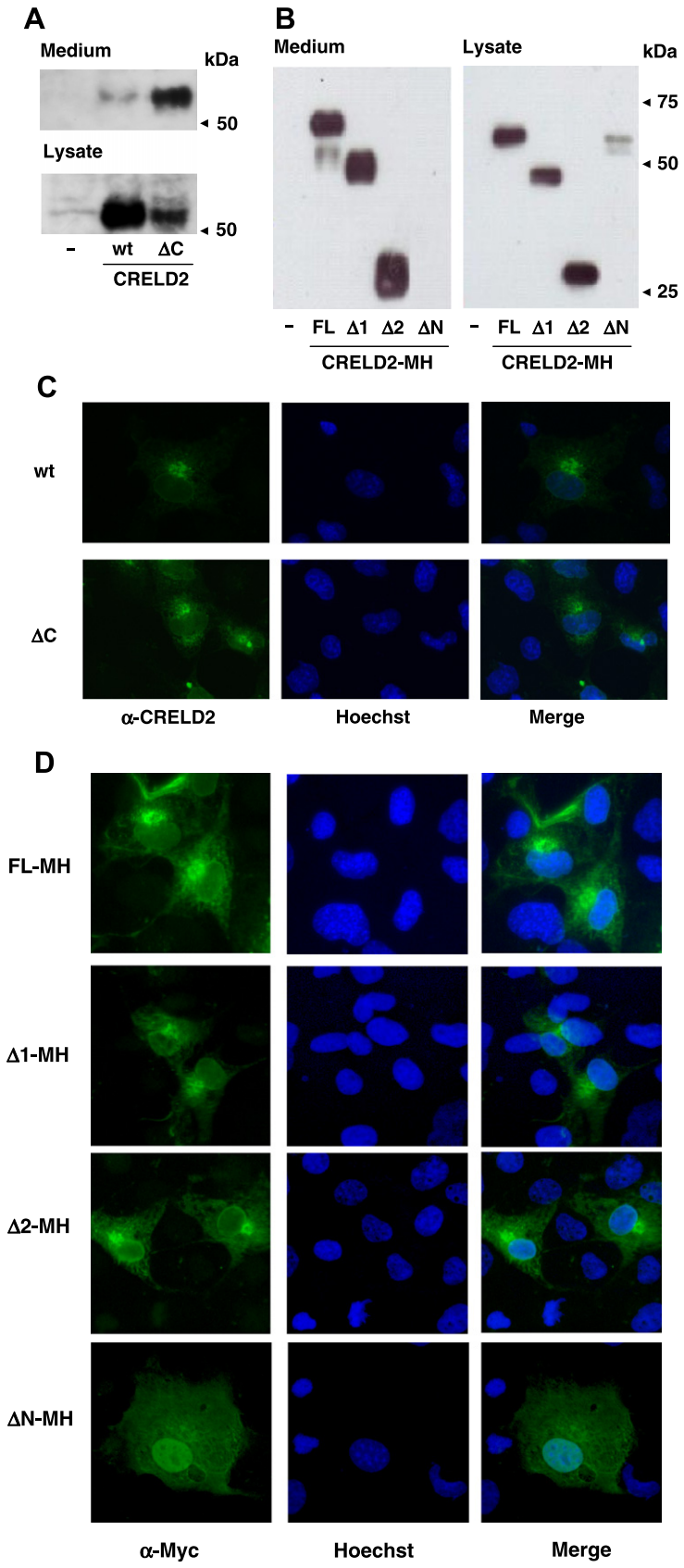

Fig. 2. Intracellular localization and secretion of wild-type and mutant CRELD2. Twenty-four hours after transfection of the indicated constructs into COS7 cells, the culture medium was replaced with fresh medium and the cells were incubated for an additional 18 h (A) or 8 h (B). The amount of CRELD2 in each lysate or culture medium was determined by western blotting as described in Section 2. COS7 cells transiently expressing the indicated type of CRELD2 were fixed and stained with anti-CRELD2 (C) or anti-Myc monoclonal antibody (D), together with Hoechst 33258 (nuclear stain) as described in Section 2.

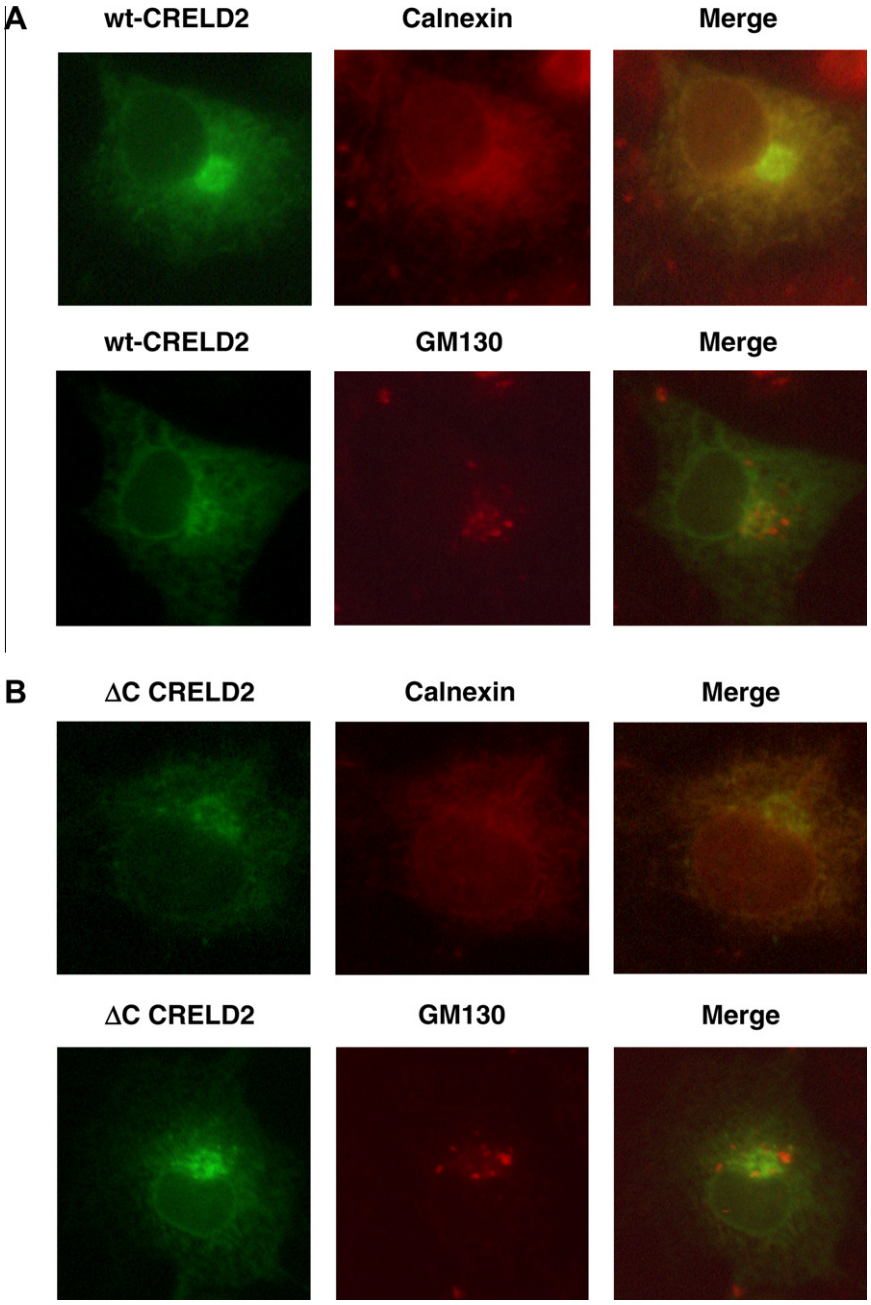

Fig. 3. Subcellular locarization of wild-type and ΔC CRELD2. COS7 cells transiently expressing wild-type (A) and ΔC CRELD2 (B) were fixed and stained with anti-CRELD2, anti-calnexin, or anti-GM130 antibody as described in Section 2.

and are similar to the C-terminal amino acids in several ER resident proteins [21]. Therefore, we prepared several CRELD2 expression constructs, which were modified at the N- and C-terminal regions (Fig. 1C), and compared their biosynthesis, localization and secretion. After HEK293 cells were transfected with wild-type (wt), lacking REDL (ΔC), Myc/His-tagged (MH) or HA-tagged (FL-HA) CRELD2, each type of CRELD2 was detected in the cells and in the culture medium, but expression of CRELD2 in the mock cells was not detected in the present condition (Fig. 1D). However, the secretion of wtCRELD2 was remarkably lower than that of other types of CRELD2, even though the cells expressing each CRELD2 were incubated for 18 h until harvested. We next transfected these CRELD2 constructs into COS7 cells and detected the intracellular localization of each protein molecule in addition to their expression in both cells and culture medium. Consistent with the expression observed in HEK293 cells, CRELD2 (ΔC) was more highly secreted from the cells relative to wtCRELD2 (Fig. 2A). These two types of CRELD2 were stained throughout the cells but their staining in the perinuclear region was relatively intensive (Fig. 2C). Similarly, each mutant CRELD2 lacking the C-terminal region (Δ1- and Δ2-MH) was secreted, as well as Myc/His-tagged full-length CRELD2 (FL-MH), and the intracellular localization of each modified protein was similar to that of wtCRELD2 (Fig. 2B and D). In contrast, another mutant CRELD2 lacking the N-terminal region with a putative signal peptide (ΔN-MH) was detected only within the cells (Fig. 2B). The intracellular localization of CRELD2 (ΔN-MH) was quite different from that of other types of CRELD2, and the ΔN-MH mutant was diffused in the cytoplasmic space (Fig. 2D). These results implied that the N-terminus of CRELD2 is essential for its translocation into the ER-Golgi apparatus, whereas the deletion of its C-terminal region had no effect on their accumulation in the perinuclear region. Then, we characterized each type

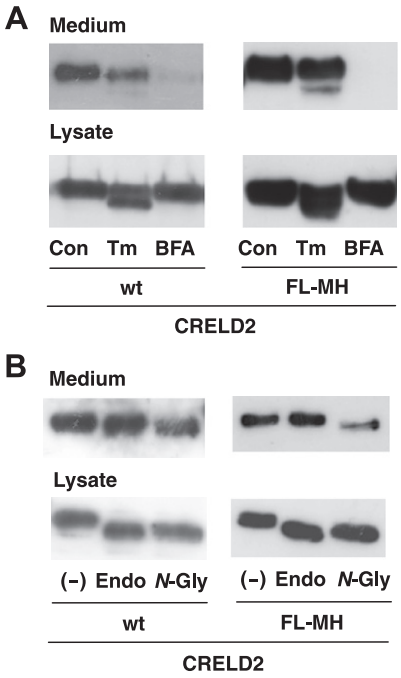

Fig. 4. Effects of Tm and BFA on the glycosylation and secretion of CRELD2. (A) Twenty-four hours after transfection of wtCRELD2 or CRELD2-MH (FL-MH) into HEK293 cells, the culture medium was replaced with fresh medium containing Tm (5 μg/ml), BFA (5 μg/ml), or vehicle and the cells were incubated for an additional 12 h (wt) or 8 h (FL-MH). (B) Samples containing wtCRELD2 or CRELD2-MH in each cell lysate and culture medium were treated with Endoglycosidase H (Endo H), *N*-Glycosidase F (*N*-Gly) or vehicle. Expression of each type of CRELD2 was determined by western blotting as described in Section 2.

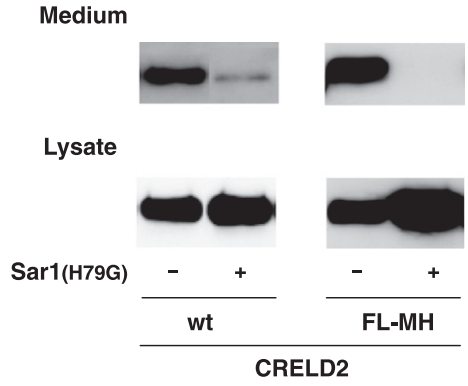

Fig. 5. Co-expression of Sar1 (H79G) reduces CRELD2 secretion. Twenty-four hours after transfection of wtCRELD2 or CRELD2-MH together with Sar1 (H79G) or empty vector into HEK293 cells, the culture medium was replaced with fresh medium and the cells were incubated for an additional 12 h (wt) or 4 h (FL-MH). The amount of CRELD2 in each lysate or culture medium was determined by western blotting as described in Section 2.

of CRELD2 in the perinuclear region by co-staining calnexin or GM130, a marker of ER or Golgi apparatus respectively. A shown in Fig. 3, the intensive signal of wild-type or ΔC CRELD2 was partially overlapped with that of GM130 in the perinuclear region. Localizations of other CRELD2, FL-, Δ1- and Δ2-MH, also showed the similar patterns in COS7 cells (Supplemental Fig. 1).

Next, we studied the effects of Tm, an inhibitor of protein glycosylation, and BFA, a reagent disrupting the Golgi apparatus, on glycosylation and secretion of CRELD2. The effects of Tm and BFA on modification and secretion of each type of CRELD2 were almost similar (Fig. 4A), though the duration of treatment of wtCRELD2-transfected cells with Tm and BFA was different from that of CRELD2-MH (FL-MH). The Tm treatment generated a lower molecular size of CRELD2, which could be an unglycosylated form. Interestingly, only a small amount of unglycosylated CRELD2 was also detected in the culture medium. The glycosylation of wtCRELD2 was also confirmed by treatment with two glycosidases, Endo H, which removes mannose sugar moieties, and *N*-Gly, which deglycosylates *N*-linked complex carbohydrates. The intracellular wtCRELD2 was sensitive to both glycosidases, whereas the carbohydrate motif of secreted wtCRELD2 was only deglycosylated by *N*-Gly (Fig. 4B). The deglycosylation of CRELD2-MH with each glycosidase also showed a similar pattern. Therefore, each type of CRELD2 is glycosylated in a similar manner, despite considerably different secretory efficiencies. These results furthermore imply that only a small part of CRELD2 is secreted into extracellular space followed by additional modification of intracellular CRELD2, but the greater part of CRELD2 retain intracellularly. On the other hand, BFA almost completely prevented each type of CRELD2 from the secretion (Fig. 4A). Taken together, these results suggest that CRELD2 is partially secreted via the ER-Golgi apparatus route and its glycosylation is not essential for the secretion.

To reveal the molecular mechanism regulating the transport of CRELD2 within the ER and Golgi apparatus, we focused on the small GTPase Sar1, which is a critical component of COPII, essential for an early step in vesicle budding and regulates vesicular transport from the ER to the Golgi apparatus [18,22,23]. Overexpression of Sar1 (H79G), which is the GTP-bound mutant and constitutive active form, dampens the export of cargo from ER [18,23]. In this study, co-transfection of Sar1 (H79G) with wtCRELD2 or CRELD2-MH markedly reduced the CRELD2 secretion and increased the accumulation of intracellular CRELD2 (Fig. 5). The effects of Sar1 (H79G) and BFA on CRELD2 secretion indicate that the COPII-mediated transport from the ER to the Golgi apparatus is a main pathway for CRELD2 secretion.

Finally, we studied the effects of GRP78 on the secretion of CRELD2, as GRP78 has been reported to regulate the degradation, transport and secretion of many kinds of proteins [24–28]. As shown in Fig. 1B, GRP78 possesses the well-known ER-resident signal sequence at its C-terminus, KDEL. Therefore, we transfected wild-type GRP78 (wtGRP78) or mutant GRP78 lacking the C-terminal KDEL sequence (ΔGRP78), together with various types of CRELD2 into HEK 293 cells (Fig. 6). Overexpression of wtGRP78 but not of ΔGRP78 remarkably increased wtCRELD2 secretion, whereas the effect of wtGRP78 on ΔC CRELD2 secretion was negligible when each band was quantified by NIH image. The secretion of CRELD2-MH was hardly affected by either type of GRP78. On the other hand, GRP78 in the culture medium was not detected in the present condition. These results indicate that the elevated amount of GRP78 would act as a positive regulator to facilitate the secretion of CRELD2 under certain pathophysiological conditions. Though further characterization of the intracellular transport, modification and secretion of CRELD2 under normal and pathological conditions are required, the excess GRP78 would mask the interaction of CRELD2 with KDEL receptor to decrease its intracellular retention and promote its secretion. The KDEL sequence in GRP78 probably has higher affinity toward the receptor than the REDL sequence in CRELD2.

We previously demonstrated that *CRELD2* is a novel ER stress-inducible gene [11], which is transcriptionally regulated by ATF6 (one of the three major ER stress sensors, ATF6 [7–10], PERK [5,8] and IRE1 [6,9]), but its molecular features and functions are hardly characterized. Ortiz *et al.* reported that human CRELD2 (hCRELD2) impairs the membrane transport of acetylcholine receptor α4/β2 [15]. They evaluated functions of two C-terminal splicing variants of hCRELD2 (α and β) with respect to their interaction with acetylcholine receptor α4. The *hCRELD2α* gene is

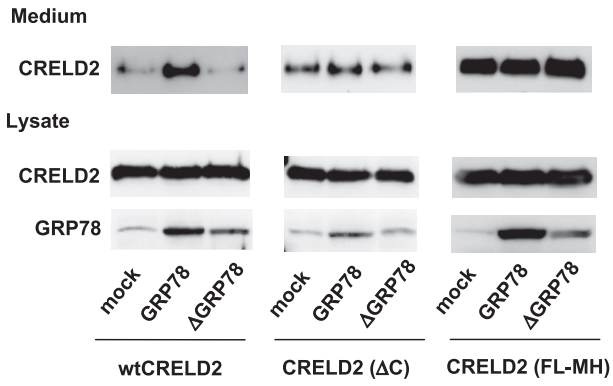

Fig. 6. Overexpression of GRP78 potentiates wtCRELD2 secretion. Twenty-four hours after transfection of wtCRELD2, CRELD2 (ΔC) or CRELD2 (FL-MH) together with either wtGRP78, ΔGRP78 or empty vector (mock) into HEK293 cells, the culture medium was replaced with fresh medium, and the cells were incubated for the appropriate time (12 h for wt, 6 h for ΔC or 4 h for FL-MH). The amount of CRELD2 in each lysate or culture medium was determined by western blotting as described in Section 2.

homologous to the mouse *CRELD2* gene and contains the sequence encoding REDL, whereas the C-terminal amino acids of hCRELD2β are different from those of hCRELD2α. Furthermore, Ortiz *et al.* showed that the WE domain (rich in tryptophan and glutamic acids) of the common N-terminal region of hCRELD2 (25–130 aa) and other regions of hCRELD2α (131–321 aa) and β (131–284 aa) interact with the cytosolic domain of acetylcholine receptor α4 (331–381 aa) [15]. Therefore, the WE domain, EGF-like domain and furin-like repeats form multiple binding regions to cytosolic α4, but the C-terminal structure of CRELD2, which we characterized in this study, is unlikely to participate in the regulation of membrane transport of acetylcholine receptor α4/β2. Very recently, Hosur et al. [16] suggest that CRELD2 would have an antagonistic activity in nicotine-induced up-regulation of acetylcholine receptor α4/β2. In their report, the induction of *CRELD2* mRNA by nicotine was not parallel with those of other ER stress-inducible genes, *GRP78* (*HSPA5*) [17,23–28] and *HERPUD1* [29]. These results suggest that CRELD2 is expected to function under ER stress-unrelated conditions and that both the secreted and intracellular CRELD2 are likely to contribute to some neuronal events via the nicotinic signaling pathways.

Among the ER stress-inducible factors, arginine-rich, mutated in early stage of tumors (Armet) [30,31] and nucleobindin 1 (NUCB1) [32–34] are reported to be secretory proteins. Armet, also called mesencephalic astrocyte-derived neurotrophic factor [35], is reported to function as a cytoprotective factor against several stimuli including neurotoxins [36], oxidative stress [37,38] and ER stress [31] both in vivo and in vitro. The intracellular NUCB1 is preferentially localized to the Golgi apparatus and interacts with S1P [34] and Gα (i1 and i3) [39,40]. Tsukumo et al. [34] demonstrated that the interaction of NUCB1 with S1P attenuates the cleavage of ATF6 and thus modulates its transcriptional activity. On the other hand, the binding of NUCB1 to Gα (i1) was reported to regulate heterotrimeric G protein trafficking and G protein-coupled receptor-mediated signal transduction pathways [40]. Like NUCB1 and Armet, CRELD2 is expected to have multiple functions involving the regulation of unidentified cellular processes both inside and outside of cells. However, we have not observed any cytoprotective features of CRELD2 in our study. Therefore, we believe that the functions of CRELD2 are not directly associated with those of Armet and NUCB1 despite the fact that the functions of the latter are not fully understood.

Transgenic mice expressing mutant matrilin-3, a structural protein of the cartilage extracellular matrix [41], and INS-1 cells overexpressing mutant insulin 2 (C96Y) derived from the Akita mouse [42] are reported to express higher amounts of *CRELD2* mRNA concomitantly with other known ER stress-inducible genes (e.g., *GRP78* and *GADD153* mRNA). However, Jariwala et al. [43] has reported that *CRELD2* is one of androgen target genes in prostate cancer cells. Therefore, we believe that CRELD2 participates in both ER stress-related and -unrelated pathophysiological conditions. In this study, we first demonstrated that a part of CRELD2 is secreted as a glycoprotein, and characterized the roles of its N- and C-terminal region in intracellular transport and secretion by overexpression of various types of CRELD2. By using cells producing higher amount of CRELD2 protein intrinsically, further characterization of CRELD2's role as a secretory factor as well as an ER- and Golgi apparatus-resident protein would provide new insights into the onset and progression of certain stress-related diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.06.029.

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